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2. Patent application number *(The Patent Office will fill in this part)* — **9820658.4**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — MEDICAL RESEARCH COUNCIL
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Patents ADP number *(if you know it)* — 5960071001

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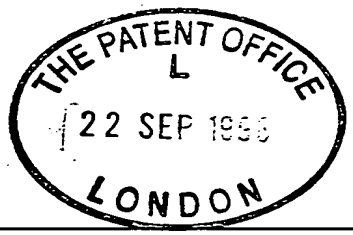

4. Title of the invention — "TREATMENT OF INFECTION"

5. Name of your agent *(if you have one)* — J A KEMP & CO

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — 14 SOUTH SQUARE
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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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TREATMENT OF INFECTION

Field of the invention

This invention relates to inhibitors of the expression and activity of a gene product and screening methods for the identification of such inhibitors. It further relates to the use of said inhibitors in the treatment of infection by an organism, such as a malaria parasite.

Background to the invention

The malaria parasite and related Apicomplexans are unusual amongst non-photosynthetic organisms in that they possess two forms of organellar DNA, typically a property of plants. One form of organellar DNA is mitochondrial DNA and the other is a 35 kb circle of DNA present in a plastid. The plastid is thought to be of prokaryotic origin. The *ycf 24* gene[1,2] which is present on the DNA of this plastid corresponds to a well conserved gene which is also found in red algal plastids[3] and various bacteria, including *E. coli*.

The plastid genome of Apicomplexans[4] is distinctive from, but broadly resembles that of the parasitic higher plant *Epifagus virginiana*[5]. It has been suggested that the *raison d'etre* for maintenance of vestigial plastid genomes is the small number of open reading frames they carry besides the highly selected set of genes dedicated to the organellar expression system[6]. Unlike Apicomplexans, *Epifagus* does not carry the *ycf 24* gene on its plastid genome, suggesting that either the vestigial organelles have different functions, or a stochastic process determines gene loss or transfer to the nucleus.

The high level of conservation of *ycf 24* (~50% amino acid identity of the putative encoded peptide product with other orthologs) suggests that it is under strong

selective pressure and likely to have a general function.

Organelle transfection is not available in Apicomplexans. Therefore "knockouts" can only be tested in surrogate organisms.

Summary of the invention

The present inventors have disrupted *ycf 24* in both *Synechocystis* sp. strain pCC6803 and *E. coli*[7]. Disruption of all the copies of the *ycf 24* gene in these organisms was found to be lethal. Disruption of some of the copies of *ycf 24* was found to give a 'ragged' phenotype in *Synechocystis* and appeared to cause a delay in or interfere in the process of septation during cell division. Thus *ycf 24* is an essential gene and appears to play a role in replication. The present invention is based on the inhibition of the expression or activity of the *ycf 24* product to inhibit the growth of an organism in vivo or ex vivo.

Thus the invention provides an inhibitor of *ycf 24* gene product expression and/or activity for use in a method of treatment of the human or animal body by therapy. Such an inhibitor is useful in particular in the treatment of infection by an organism, such as a malaria parasite. The invention also provides a method of inhibiting the growth of an organism comprising contacting the organism ex vivo with an inhibitor of *ycf 24* gene product expression and/or activity.

Screens may be carried out to identify the inhibitor. Accordingly the invention provides a method of identifying a compound that inhibits the growth of an organism comprising

(i) contacting a test compound with the *ycf 24* gene product, and

(ii) determining whether the test compound inhibits the

activity of or binds to the product, any such binding or inhibition being indicative that the compound inhibits the growth of the organism.

The invention also provides a method of identifying a compound that inhibits the growth of an organism comprising

(i) contacting a test compound with a test construct comprising a *ycf 24* promoter operably linked to a coding sequence,

(ii) determining whether the test compound inhibits expression driven by the promoter, any such inhibition being indicative that the compound inhibits the growth of the organism.

The invention further provides a pharmaceutical composition comprising an inhibitor of the invention and a pharmaceutically acceptable carrier or diluent.

As noted above the inhibitor may be used to treat an infection by an organism and therefore the invention provides a method of treating a host suffering from an infection by an organism, which method comprises administering to the host a therapeutically effective amount of an inhibitor of *ycf 24* product expression and/or activity.

Brief description of the drawings

The invention is illustrated by the accompanying drawings in which:

Fig.1 shows phenotypic characters of wild type (WT), ragged transformant (R) and smooth mutant forms (S) of *Synechocystis* sp. : a) Colony shape and size; an enlargment is inserted in R, b) Scanning electron micrographs of whole cells - bar = 10μm, c) Cross-sections showing plaques - arrow, d) Whole cells with and without DAPI-staining. Arrows point to mis-segregation.

Fig.2 shows a Southern blot of DNA from

Synechocystis sp., restricted with *Hin*d III and hybridized with wild type (WT) *ycf* 24. The sizes expected for restriction fragments from WT (lane 4) and clones of heteroplasmic transformants (lanes 1-3) are given in the diagram below.

Detailed description of the invention

The inhibitor can be used to inhibit the growth of a unicellular or multicellular organism in vivo or ex vivo. The organism may be a prokaryote or a eukaryote. The organism comprises the *ycf 24* gene. In the case of a eukaryote the nuclear genome of the organism may contain the *ycf 24* gene or the organism may contain an organelle which contains DNA comprising the *ycf 24* gene. Such an organelle is generally of prokaryotic origin, for example algal origin, and may be a plastid.

The organism may be a protozoa, such as an Apicomplexan, for example of clade plasmodium, piroplasm, sarcocystan or coccidium. The organism may be *Plasmodium falciparum*. The organism may be an alga. The organism may be a bacterium, such as a cyanobacterium, mycobacterium or *E. coli*. The organism may be free-living or a pathogen of humans and/or animals. It may be an intracellular or extracellular pathogen.

The *ycf 24* gene product is generally one which can be expressed from the coding region of:
(a) the polynucleotide sequence of SEQ ID NO: 1, 2 or 3 or a fragment thereof; or
(b) polynucleotides which can selectively hybridise to the coding region of (a) of a fragment thereof; or
(c) polynucleotides which, but for the degeneracy of the genetic code, would hybridise to (a) or (b).

The polynucleotides of (b) may selectively hybridise under conditions of medium to high stringency, for example 0.03M NaCl and 0.03M sodium citrate at from 50 to

60 degrees centigrade.

Thus the polynucleotide from which the *ycf 24* product is expressed generally has at least 70%, preferably at least 95, 97 or 99% sequence identity to the coding sequence of SEQ ID NO: 1, 2, or 3 over a region of at least 20, preferably at least 30, for instance at least 46, 60 or 100 or more contiguous nucleotides.

The *ycf 24* product generally has at least 50% sequence identity to the amino acid sequences shown is SEQ ID NO: 1, 2, or 3, preferably at least 80 or 90% and more preferably at least 95, 97 or 99% over a region of at least 20, preferably at least 30, for instance at least 46, 60, 100 or more contiguous amino acids. The term *ycf 24* product includes fragments of the amino acid sequences of SEQ ID NO: 1, 2 or 3 or of the homologous sequences discussed above. Suitable fragments will be at least 5, 10, 20 or 40 amino acids in size, for example at least 60 or 100 amino acids in size. Generally the *ycf 24* product has *ycf 24* product activity, and would generally be able to complement the activity of a naturally occurring *ycf 24* product.

Any suitable inhibitor of *ycf 24* product activity may be used in the present invention. The inhibitor may bind in a reversible or irreversible manner. An irreversible inhibitor would dissociate very slowly from the *ycf 24* product because it would be very tightly bound, either covalently or non-covalently. Reversible binding, in contrast with irreversible binding, is characterised by a rapid dissociation of the *ycf 24* product-inhibitor complex.

As noted above the invention provides a method of identifying a compound that inhibits the activity of *ycf 24* product. *ycf 24* product for use in this method can

be obtained, for example, recombinantly by any method known in the art. The nucleotide sequences of the *ycf 24* genes of *Plasmodium falciparum, Synechocystis* Sp strain pCC6803 and *E.coli* are provided herein as SEQ ID NOs 1, 2 and 3 respectively. The *ycf 24* genes of other species may be identified by searching in databases for genes homologous to SEQ ID NO: 1, 2 or 3. Nucleotides with the same sequence as genes identified in a database can be made and expressed using routine methods which are known in the art. Alternatively *ycf 24* genes can be identified by probing genomic or cDNA libraries with nucleotides comprising the *ycf 24* gene or a fragment of the *ycf 24* gene and then isolating and sequencing the nucleotides identified by the probe.

Any suitable format may be used for identifying the inhibitor of the activity. In the method the *ycf 24* product is generally in a suitable buffer, which includes any suitable biological buffer that can provide buffering capability at a pH conducive to the reaction requirements of the *ycf 24* product. The *ycf 24* product may be in conditions which are similar to the physiological conditions in which it occurs naturally.

In the method the *ycf 24* product may be in inside a cell or outside a cell. The cell may be the cell in which the *ycf 24* product naturally occurs, or a cell in which the *ycf 24* product is expressed recombinantly. The cell may be treated with agents which permeabilise the cell surface allowing test substances to enter the cell more readily. The *ycf 24* product used in the method may be in the form of an extract from such cells.

The method may determine whether a test compound is able to bind the *ycf 24* product, such as in a specific manner. This method may comprise allowing the *ycf 24* product to bind to a test compound and measuring the

amount of binding that occurs, such as by measuring the amount of *ycf 24* bound to the compound. The amount of binding may be determined by measuring a characteristic of the *ycf 24* product that changes upon binding, such as spectroscopic changes.

The assay format may be a 'band shift' system. This involves determining whether a test compound advances or retards the *ycf 24* product on gel electrophoresis relative to the *ycf 24* product in the absence of the compound.

The method may be a competitive binding assay. This determines whether the test compound is able to inhibit the binding of the *ycf 24* product to a substance which is known to bind to the *ycf 24* product, such as an antibody specific for product. This method allows test compounds to be identified which are 'analogues' of ycf 24 product.

The competitive binding system may comprise
(i) incubating the *ycf 24* product with a test compound and a labelled reference compound that is known to bind the product;
(ii) determining the amount of the labelled reference compound that is bound to the product; and
(iii) comparing the amount of bound labelled reference compound determined in step (ii) with the amount of said compound that binds to the product in the absence of the test compound;
wherein any reduction in the binding of the labelled reference compound in the presence of the test compound compared to the binding in the absence of the test compound shows that the test compound is competing with the reference compound for binding to the product. This indicates that the test compound could be an inhibitor of *ycf 24* product activity.

The amount of the labelled reference compound bound to the *ycf 24* product may be measured directly or

indirectly. A direct measurement may be carried out by removing assay mixture containing the unbound labelled reference compound and measuring the amount of label that is in the product fraction. Alternatively, the amount of labelled reference compound bound to the product could be determined indirectly by measuring the amount of label remaining in the assay solution after removal of the product fraction, which will be inversely related to the amount that has bound to the product.

In a competitive binding assay system, the *ycf 24* product may be immobilised on a solid support or may be in solution. The use of immobilised product has the advantage that, after the binding reaction is complete, the product/labelled reference compound complex may be separated from the labelled reference compound that remains in solution by simply removing the solution away from the solid support. If, on the other hand, the product is not immobilised during the assay but rather is in solution, then it will generally be necessary to devise a means for separating the product/labelled reference compound complex from the uncomplexed reference compound before measuring the amount of label. Such separation could be achieved, for example, by precipitating the product using an antibody to the product or by using a non-specific precipitation technique.

Suitable labels for use in the assay systems according the invention are well-known in the art. The reference compound which is know to bind the *ycf 24* product may be an antibody.

An antibody to *ycf 24* product may be produced by raising antibody in a host animal against the whole *ycf 24* product or an antigenic epitope thereof (hereinafter "the immunogen"). Methods of producing monoclonal and polyclonal antibodies are well-known. A

method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified. A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein, Nature 256, 495-497, 1975).

An immortalized cell producing the desired antibody may be selected by a conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an allogenic host or immunocompromised host. Human antibody may be prepared by *in vitro* immunisation of human lymphocytes, followed by transformation of the lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier. The carrier molecule is typically a physiologically acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

Any suitable inhibitor of expression of *ycf 24* product may be employed in the present invention. Preferably the inhibitor is a specific inhibitor of transcription from the *ycf 24* gene, and does not inhibit transcription from other genes.

The inhibitor may inhibit transcription or

translation of *ycf 24* product. The inhibitor may bind to the *ycf 24* gene either 5' to the coding sequence, to the coding sequence or 3' to the coding sequence. Thus the inhibitor may bind to the *ycf 24* promoter, and inhibit the initiation of transcription. The inhibitor may bind and inhibit the action of a protein which is required for transcription from the *ycf 24* gene. The inhibitor may bind to the untranslated or translated regions of the *ycf 24* mRNA. This could prevent the initiation of translation. Alternatively the inhibitor could bind to a protein which associates with the untranslated region and prevent the protein associating with the untranslated region.

Any suitable assay format may be used for identifying an inhibitor of *ycf 24* expression. The invention provides a method for identifying an inhibitor of *ycf 24* expression comprising:

(i) contacting a compound with a nucleic acid comprising a *ycf 24* promoter operably linked to a coding sequence; and

(ii) determining whether the compound inhibits expression driven by the promoter.

Generally this method is carried out in conditions which in the absence of the test compound lead to expression of the coding sequence from the nucleic acid. The nucleic acid may also comprise other untranscribed or untranslated regions of the *ycf 24* gene. The coding sequence typically encodes a protein that is able to act as a reporter of expression. The assay may be carried out in a cell which harbours the nucleic acid. The substance may be tested with any other known promoter to test the possibility that the test substance is a general inhibitor of gene expression.

Any reporter polypeptide may be used, for example GUS or GFP. GUS is assayed by measuring the hydrolysis of a suitable substrate, for example 5-bromo-4-chloro-3-

indolyl-β-D-glucoronic acid (X-gluc)or 4-methylumbelliferyl-β-glucuronide (MUG). The hydrolysis of MUG yields a product which can be measured fluorometrically. GFP is quantified by measuring fluorescence at 590nm after excitation at 494nm. These methods are well known to those skilled in the art.

Alternatively the coding sequence may be the *ycf 24* coding sequence itself, or a fragment of this sequence. The expression of the *ycf 24* product may be measured by for example, Northern/RNA blotting, Western/antibody blotting, RNA in situ hybridization or immunolocalisation. Thus the method for identifying an inhibitor of expression of *ycf 24* product may be performed on a cell which naturally harbours the *ycf 24* gene, or an extract from such a cell.

Suitable candidate substances which may be inhibitors and which can be tested in the methods discussed above include antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies and CDR-grafted antibodies) which are specific for *ycf 24* product. Furthermore, combinatorial libraries, defined chemical identities, peptide and peptide mimetics, oligonucleotides and natural product libraries , such as display libraries (e.g. phage display libraries) may also be tested. The candidate substances may be chemical compounds. Batches of the candidate substances may be used in an initial screen of, for example, ten substances per reaction, and the substances of batches which show inhibition tested individually.

Antisense inhibitors

As noted above the expression of *ycf 24* in a cell may be reduced by the presence in that cell of a compound which can bind to the *ycf 24* mRNA. Therefore a polynucleotide

which is capable of hybridizing to *ycf 24* mRNA can constitute an appropriate inhibitor of *ycf 24* expression.

The polynucleotide may be antisense to the *ycf 24* mRNA. Such a polynucleotide may be capable of hybridising to *ycf 24* mRNA and may thus inhibit the expression of *ycf 24* product by interfering with one or more aspects of *ycf 24* mRNA metabolism including processing, translation and metabolic turnover.

The antisense polynucleotide may be RNA. This may hybridise to the *ycf 24* mRNA to form an RNA-RNA duplex which may cause direct inhibition of translation and/or the destabilization of the target message, for example, rendering susceptibility to nucleases.

Such a polynucleotide may hybridize to all or part of the *ycf 24* mRNA. Typically the antisense polynucleotide hybridizes to the ribosome binding region or the coding region of the *ycf 24* mRNA. The polynucleotide may be complementary to all of or a region of the *ycf 24* mRNA. For example, the polynucleotide may be the exact complement of all or a part of *ycf 24* mRNA. However, absolute complementarity is not required and polynucleotides which have sufficient complementarity to form a duplex having a melting temperature of greater than 20°C, 30°C or 40°C under physiological conditions are particularly suitable for use in the present invention. The polynucleotide may be a polynucleotide which hybridises to the *ycf 24* mRNA under conditions of medium to high stringency such as 0.03M sodium chloride and 0.03M sodium citrate at from about 50 to about 60 degrees centigrade.

It is preferred that the polynucleotide hybridizes to all or part of the region of the *ycf 24* mRNA corresponding to the coding sequence defined by nucleotides 26 to 1435 of SEQ ID NO:1. However, the

polynucleotide may hybridise to all or part of the 5'- or 3'-untranslated region of the mRNA. The polynucleotide will typically be from 6 to 40 nucleotides in length. Preferably it will be from 12 to 20 nucleotides in length. The polynucleotides may be at least 40, for example at least 60 or at least 80, nucleotides in length and up to 100, 200, 300, 400, 500, 600 or 700 nucleotides in length or even up to a few nucleotides, such as five or ten nucleotides, shorter than SEQ ID NO: 1, 2 or 3.

When the polynucleotide is an antisense RNA it may be expressed in a cell from a recombinant replicable vector. Such a replicable vector comprises a polynucleotide which when transcribed gives rise to antisense RNA.

Thus the antisense polynucleotide may be provided by delivering such a vector to the cell and allowing transcription from the vector to occur. Such a vector is also understood to an inhibitor of *ycf 24* expression in this specification. Generally on the vector the polynucleotide giving rise to the antisense RNA is operably linked to a control sequence which is capable of providing for the transcription of the polynucleotide giving rise to the antisense RNA. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a sequence giving rise to an antisense RNA is ligated in such a way that transcription of the sequence is achieved under conditions compatible with the control sequences.

The vector may be for example, a plasmid or virus vector provided with an origin of replication, optionally a promoter for transcription to occur and optionally a regulator of the promoter. The vector may contain one or more selectable marker genes, for example an ampicillin

resistance gene in the case of bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used in vitro, for example for the production of antisense RNA or used to transfect or transform a host cell. The term 'host cell' refers either to the organism whose growth it is desired to inhibit, or a human or animal cell which can be infected by the organism. Thus the host cell may be an infected cell. The vector may also be adapted to be used in vivo, for example in a method of gene therapy.

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, mammalian promoters, such as b-actin promoters, may be used. Tissue-specific promoters, in particular neuronal cell specific promoters (for example the tyrosine hydroxylase (TH), L7, or neuron specific enolase (NSE) promoters), are especially preferred. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are readily available in the art. Preferred promoters are tissue specific promoters such as the casein gene promoter.

The vector may further include sequences flanking the polynucleotide giving rise to antisense RNA which comprise sequences homologous to eukaryotic genomic sequences, preferably mammalian genomic sequences, or viral genomic sequences. This will allow the introduction of the polynucleotides of the invention into the genome of eukaryotic cells or viruses by homologous recombination. In particular, a plasmid vector comprising the expression cassette flanked by viral sequences, for example HSV1 or

HSV2 sequences, can be used to prepare a viral vector, for example an HSV vector, suitable for delivering the polynucleotides of the invention to a mammalian cell. Other examples of suitable viral vectors include retroviruses, including lentiviruses, adenoviruses and adeno-associated viruses. Gene transfer techniques using these viruses are known to those skilled in the art. Retrovirus vectors for example may be used to stably integrate the polynucleotide giving rise to the antisense RNA into the host genome. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression.

Antisense polynucleotides may be chemically modified. This may enhance their resistance to nucleases and may enhance their ability to enter cells. For example, phosphorothioate oligonucleotides may be used. Other deoxynucleotide analogs include methylphosphonates, phosphoramidates, phosphorodithioates, N3'P5'-phosphoramidates and oligoribonucleotide phosphorothioates and their 2'-O-alkyl analogs and 2'-O-methylribonucleotide methylphosphonates.

Alternatively mixed backbone oligonucleotides (MBOs) may be used. MBOs contain segments of phosphothioate oligodeoxynucleotides and appropriately placed segments of modified oligodeoxy- or oligoribonucleotides. MBOs have segments of phosphorothioate linkages and other segments of other modified oligonucleotides, such as methylphosphonate, which is non-ionic, and very resistant to nucleases or 2'-O-alkyloligoribonucleotides.

An inhibitor of *ycf 24* expression and/or activity is one which produces a measurable reduction in *ycf 24* expression and/or activity in the methods described above. Preferred substances are those which inhibit *ycf 24* expression and/or activity by at least 10%, at least 20%, at least 30%, at least 40% at least 50%, at least 60%, at

least 70%, at least 80%, at least 90%, at least 95% or at least 99% at a concentration of the inhibitor of 1µg ml^{-1}, 10µg ml^{-1}, 100µg ml^{-1}, 500µg ml^{-1}, 1mg ml^{-1}, 10mg ml^{-1} or 100mg ml^{-1}. The percentage inhibition represents the percentage decrease in expression/activity in a comparison of assays in the presence and absence of the test substance. Any combination of the above mentioned degrees of percentage inhibition and concentration of inhibitor may be used to define an inhibitor of the invention, with greater inhibition at lower concentrations being preferred.

When used in vivo or in vitro the inhibitor inhibits the growth of the organism. Generally the inhibitor will decrease the rate of cell division of the organism. Generally the inhibitor will decrease the number of divisions that occur in a unit of time by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% at a concentration of inhibitor of 1µg ml^{-1}, 10µg ml^{-1}, 100µg ml^{-1}, 500µg ml^{-1}, 1mg ml^{-1}, 10mg ml^{-1} or 100mg ml^{-1}.

If the organism is one in which the *ycf 24* gene is present in an organelle then the inhibitor generally decreases the rate of division of that organelle. The inhibitor may also disrupt cell or organelle division in other ways, such as causing abnormal septation or mis-segregation of DNA. The inhibitor may also disrupt the structure of the organism, such as giving the organism a 'ragged' appearance. The inhibitor may cause the death of the organism.

Candidate substances which show activity in assays such as those described herein can then be tested on the organism in question. The inhibitor may or may not be toxic towards humans or animals. The inhibitor may thus be used as an antibiotic.

The inhibitors of *ycf 24* expression and/or activity or the vectors described above may be used to treat infections by an organism and, in particular, malaria. The

condition of a patient suffering from an infection can therefore be improved by administration of such an inhibitor. A therapeutically effective amount of such an inhibitor may be given to a human patient in need thereof.

The formulation of inhibitor or vector for use in preventing or treating infection by an organism will depend upon factors such as the nature of the substance identified, whether a pharmaceutical or veterinary use is intended, etc. In order to be administered to a patient, the compound will be provided in the form of a pharmaceutical composition containing the inhibitor or vector and a pharmaceutically acceptable carrier or diluent. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. Typical oral dosage compositions include tablets, capsules, liquid solutions and liquid suspensions. For example it may be formulated for parenteral, intravenous, intramuscular, subcutaneous, transdermal or oral administration.

The dose of inhibitor may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. A physician will be able to determine the required route of administration and dosage for any particular patient. A suitable dose may however be from 0.1 to 100 mg/kg body weight such as 1 to 40 mg/kg body weight, for example, to be taken from 1 to 3 times daily.

The oral route is preferred because this is generally the most convenient route for a patient to take regular doses of the compound without the assistance of a physician. However, the inhibitor or vector may be administered by direct injection into the site to be treated.

The inhibitor or vector may be used in either the

treatment of an existing infection by the organism or in the prevention of such an infection from occurring in the first place.

The vectors giving rise to antisense RNA of the invention may be administered directly as a naked nucleic acid construct. Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques, for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). These agents may also enhance the uptake of the constructs by the organism.

The pharmaceutical composition may be formulated to aid delivery of the inhibitor or vector to the organism whose growth is to be inhibited. In particular it may be formulated to deliver the inhibitor or vector to the cell type which is infected or to the compartment in the human or animal cell which is infected by the organism. It may be formulated to deliver the inhibitor or vector to the organelle in the organism where *ycf 24* product or mRNA is present.

When the pharmaceutical composition comprises viral vector for gene therapy it may be administered in such a way that the vector is incorporated into cells at an appropriate area. The amount of virus administered is in the range of from 10^4 to 10^8 pfu, preferably from 10^5 to 10^7 pfu, more preferably about 10^6 pfu for herpes viral vectors and from 10^6 to 10^{10} pfu, preferably from 10^7 to 10^9 pfu, more preferably about 10^8 pfu for adenoviral vectors. When injected, typically 1-2 ml of virus in a pharmaceutically acceptable suitable carrier or diluent is administered. When the polynucleotide is administered as a naked nucleic acid, the amount of nucleic acid administered is typically in the range of from 1 µg to 10 mg.

Where the polynucleotide giving rise to the antisense RNA is under the control of an inducible promoter, it may only be necessary to induce gene expression for the duration of the treatment. Once the condition has been treated, the inducer is removed and expression of the polypeptide of the invention ceases. This will clearly have clinical advantages. Such a system may, for example, involve administering the antibiotic tetracycline, to activate gene expression via its effect on the tet repressor/VP16 fusion protein.

The use of tissue-specific promoters will be of assistance in the treatment of disease using the vectors of the invention. It will be advantageous to be able express inhibitors of *ycf 24* in only the relevant affected cell types, especially where such inhibitors are toxic when expressed in other cell types.

When the inhibitor is used to inhibit the growth of an organism ex vivo the organism may or may not be a pathogen of humans or animals. The inhibitor may be used to treat land, water or food containing the organism.

The following Examples illustrate the invention:

Example 1

Gene disruption of *ycf* 24, transformation and selection

A central segment of the wild type gene (slr0074) from *Synechocystis* sp., strain pCC6803, (delineated by two *Hin*dIII sites ~1.0 kb apart) was amplified from genomic DNA by PCR using two oligonucleotide primers based on the known sequence (Accession no. S76598): 5' CTC CGC CCC TAA GCA AAG TAA GGA A and 5' TGA TCC TCG CCA ATT TTG GAA GTG GA. The amplified fragment was restricted with *Hin*dIII and cloned into pUC9 to give pUC9/slr . The sequence of both ends of the insert was confirmed in recombinants grown in Epicurian SURE cells. To disrupt the gene, a kanamycin resistance gene (k^r) isolated from a modified bluescript

vector (pBSHdSp1, was blunt-ended with the Klenow fragment and cloned into a unique *Xba*I site in the *ycf* 24 insert to give pUC9/slr/k^r. Recombinants in XL1-blue MRF' cells were selected using kanamycin. Disruption of the *ycf* 24 insert was confirmed by sequencing the junction regions.

Wild type (WT) cells of *Synechocystis* sp. strain PCC 6803 were transfected by incubation with light at 30°C for 4 hr with vector DNA carrying the disrupted *ycf* 24 gene (1-5 µg in 10 µl water). Selection and growth were carried out on BG11 plates under photo-autotrophic conditions at 30°C. Several rounds of replication were allowed over 3 days before recombinants were selected by overlaying with kanamycin (50 µg/ml). This killed most of the cells but transformants emerged after a delay. Every 10-14 days thereafter, transformants were picked and re-plated to allow segregation of mutant cells. For growth comparisons in the absence of selection, WT cells were plated at the same time on BG11 (their growth was inhibited totally by kanamycin at 50 µg/ml).

Example 2

Gene Disruption in *E.coli*

Disruption of *ycf* 24 in *E. coli* was carried out along similar lines, but in this case disruption was with the *aad*A gene for streptomycin resistance (S^r). *ycf* 24 from *E. coli* was amplified using primers based on the accession no. D90811 with added terminal restriction sites: 5' GAG CTC GGA ATT CGC ATG TGG CGA AAG and 3' GAG CTC GGG ATC CGA CGC TGT GTT CAA G. The *E.coli aad*A gene was introduced at a *Bsg*1 restriction site near the centre of *ycf* 24 and cloned into the bluescript vector pBSKS$^+$ The construct was linearized for transformation and to allow homologous recombination in *E. coli* INValphaF' One Shot cells. The linearized recombinant plasmid pBSKS$^+$/*ycf*24/S^r was

transfected into *E. coli* by heat shock but no homologous recombinants were found; a few antibiotic resistant colonies were recovered that carried circular episomes likely to have arisen from re-ligation of the construct.

Example 3

Characterising the Synechocytis transformants

WT *Synechocystis* and transfectants carrying an homologous form of *ycf* 24 interrupted by a kanamycin resistance gene (k^r) were plated on BG-11 agar. The WT cells formed colonies visible to the naked eye within 4 days, whereas it took 2 weeks for transformants to appear under selection with kanamycin (50 μg ml^{-1}). Colonies of transformants were small and "ragged" in outline in contrast to the larger, smooth-contoured WT colonies (Fig. 1a). DNA extracted from cloned transformants was hybridized in Southern blots with WT *ycf* 24 and found to be heteroplasmic, the copy numbers of the disrupted and WT forms of *ycf* 24 being approximately equal as judged from the intensity of the hybridization signals (Fig. 2) . Relaxation of kanamycin selection on heteroplasmic transformants resulted in vigorously growing, smooth-contoured colonies - "revertants", confirming that the heteroplasmic state was growth-limiting. These observations were confirmed by additional transformations, including one using disruption with a spectinomycin resistance gene instead of kanamycin. Transformants homoplasmic for the disrupted form of *ycf* 24 were never recovered, even after prolonged selection with kanamycin at 50 or 100 μg ml^{-1} and homoplasmy is inferred to be lethal.

Longer-term selection of "ragged" transformants on kanamycin also gave rise to rapidly growing colonies with smooth contours. However, hybridization on Southern blots showed these remained heteroplasmic for the disrupted gene and we refer to these as "smooth" suppressors. DNA from

cloned "smooth" suppressors was used to transform both WT and "ragged" heteroplasmic cells. In both cases, kanamycin selection yielded mostly "ragged" colonies but small numbers (<1%) of smooth, rapidly growing colonies were found, unlike in the original transfections.

The cells were examined by electron microscopy. Pelleted cells were pre-fixed in 0.5% neutral glutaraldehyde in 100mM cacodylate buffer, pH 7.0 for 30 min at room temperature. After gentle centrifugation, the pelleted cells were fixed in 2-3% glutaraldehye buffer and thin sections were prepared using standard procedures. For scanning electron microscopy (SEM), cells were spread on glass slips coated in poly-L-lysine (100 μg ml^{-1}) and fixed in 2% glutaraldehyde. Post fixation samples were washed in ethanol and rehydrated by immersion in hexamethyl-disilizane. After overnight dessication, samples were sputter-coated with 20 nm gold (for sample conductivity) and examined by SEM.

Electron micrographic sections showed ~80% of cells from "ragged" colonies (>100 cells examined) had electron-translucent plaques surrounding the phycobilisomes in the thylacoid membranes (Fig. 1c). The number of cells containing plaques fell to ~45% upon relaxation of kanamycin selection pressure, coincident with reversion to normal colony morphology. Because similar plaques were found in ~4% WT cells, we suggest they indicate "sick" cells rather than a direct phenotypic change following disruption of *ycf* 24.

By contrast, examination of the gross morphology of cells from "ragged" colonies by scanning electron microscopy showed a significant accumulation of cells (~50%) in the last stages of cell division ($p = <0.001$), suggesting a defect in cytokinesis (Table 1 and Fig.1b). Relaxation of kanamycin selection again saw the proportion of dividing cells in the transformed cell population return

to one resembling WT . A high proportion (~60%) of the "smooth" suppressor cells also accumulated at a late stage of cytokinesis, with occasional small chains of incompletely separated cells. Smooth suppressors still contained electron-opaque plaques and electron micrographic sections confirmed that suppression increased the frequency of abberant septation in dividing cells.

Table 1

	% Fission			
Cell type *1	Early stage	Late stage	Total	P (X^2) *3
WT	10	9±1	19	-
Ragged	21 ±6	34±1	55	<0.001
Revertant *2	8	4	12	0.9
Smooth	20	41	61	<0.001

*1 Nos. cells counted = >100/group (mean data from 2 exp.)
*2 Ragged transformants grown without kanamycin selection
*3 Chi-square comparison with WT

DNA was stained with 4',6-diamindino-2-phenylindole (DAPI) at 0.01µg ml^{-1}, following a protocol similar to that described[15]. When stained with DAPI, the nucleoids of WT cells were highly uniform in appearance (Fig.1d). Most WT cells (~82%) were not in division and only a few dead cells (unstained) were present. DAPI-staining of cells from "ragged colonies' was much more variable, ranging from cells that looked like WT to a large number (~50%) of dead or dying cells that stained less intensely. The latter included >50% of those in division. Although nucleoid segregation had occurred in many of these cells, in ~40% the DNA had mis-segregated (ie. one daughter cell was without DNA or carried less DNA as judged by staining

intensity); altered autofluorescence under UV light indicated these were dying cells. DAPI-stained "smooth" suppressants also were distinctive. About half of the cells (both dividing and non-dividing) carried normal amounts of DNA but occasional cells either had no DNA (~1% dead), had undergone asymmetric DNA segregation (~1%), or had about twice as much DNA (~1%) as WT. The cells in this last group were larger than the rest (Fig. 1d). Coulter counter analysis of the size distribution of the different populations (data not shown) confirmed that the numbers of small and large cells had substantially increased in both the "ragged" and smooth isolates compared with WT. The amount of DNA per cell corresponded generally with cell size, excepting dead cells.

Example 4

Over-expression of *ycf* 24 in *E.coli*

The effect of over-expressing *ycf* 24 in *E. coli* also was examined using into the expression vector pMAL-c2. Gene amplification was carried out with primers designed to give a 5' *Eco* RI site and a 3' *Pst* I site, and the product was cloned into the pMAL-c2 and p2 expression vectors (New England Biolabs). Cultures of transfectants growing exponentially in Terrific broth containing ampicillin (50 $\mu g\ ml^{-1}$) were induced with 0.5 mM IPTG. Transformants expressing fusion proteins after induction at 37°C with IPTG were detected by Western blotting. Filamentation was marked in such transformants. The gene was also over-expressed in the periplasmic expression vector pMAL-p2 . One such transformant over-expressed a soluble, truncated fusion protein causing stasis of cultures within 1 hr at 37°C and cell death. The fusion protein appeared to have a C-terminal deletion.

Conclusions

We conclude that disruption of *ycf* 24 in *Synechocystis* sp., by homologous recombination is lethal, except in cells with a heteroplasmic genotype; these nevertheless have severely impaired colony growth. The ragged appearance (sectoring) attributed to cell death is not likely to be due to loss of k^r by segregation as this does not occur following its random insertion into the genome. Accumulation of heteroplasmic cells at a late stage in cytokinesis, suggests delayed or incomplete septation and many "dividing cells" died. DAPI-staining also showed mis-segregation and loss of DNA. This finding contrasts with the only other *ycf* 24 knockout we know of, recorded in the Cyanomutant database, where no phenotype was observed. It is possible this difference is related to our finding that revertants emerged rapidly upon relaxation of kanamycin selection, and that "smooth" suppressor mutants also emerged under continuous kanamycin selection. Smooth suppressants are presumed to be the result of compensatory mutation(s), the character(s) being transformable and able to confer viability to heteroplasmic transformants without restoring them to WT. The multiple-septation evident in "smooth" suppressants may be in keeping with their rapid growth rate[8], but asymmetric septation also occurred resulting in small chains of deformed cells.

Over-expression of *ycf* 24 in *E. coli* also blocked cytokinesis. The possibly that this was elicited by the SOS response[9] remains to be investigated, but cytoplasmic expression, unlike periplasmic expression, did not cause cell death over a period of 2-3 hr after induction. Interestingly, *ycf* 24 carries an intein in some species of mycobacteria[10], a feature long known of some genes concerned with DNA regulation (eg. *rec*A and DNA polymerase[11]).

Database searches with the putative peptide encoded by *ycf* 24 for homologies with proteins of known function

showed no significant "hits", but protein folding motifs were found (using the programme THREADER[12]) with significant homology to those of an O-glycosyl hydrolase - namely, alpha amylase.

It follows from our observations that *ycf* 24, an essential house-keeping gene essential at a late stage of the prokaryotic cell cycle, is not the *raison d'etre* of the vestigal apicomplexan plastid genome, ie. it does not encode the primary function. Yet retention of *ycf* 24 on this highly degenerate plastid genome can be reconciled with the necessity for strict control of DNA segregation and plastid replication. In the parasites examined so far, each cell carries a single plastid with only a few copies of the 35 kb plastid genome - estimates are ~1-3 copies/cell in *P. falciparum*, whereas *Toxoplasma gondii* has >5 copies[13]. Replication of plastid DNA in apicomplexans is essential for cell survival and has been shown to be vulnerable to antibiotic interference[13,14].

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SEQUENCE LISTING

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65                  70                  75                  80
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385                 390                 395                 400
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Gln Cys Asp Ser Met Leu Ile Gly Ala Asn Cys Gly Ala His Thr Phe
            420                 425                 430
ccg tat gtt gag tgt cgt aac aat agt gcg caa ctg gaa cac gag gca  1344
Pro Tyr Val Glu Cys Arg Asn Asn Ser Ala Gln Leu Glu His Glu Ala
        435                 440                 445
acg aca tca cgt att ggt gaa gat caa ctg ttt tac tgc ctg caa cgc  1392
Thr Thr Ser Arg Ile Gly Glu Asp Gln Leu Phe Tyr Cys Leu Gln Arg
    450                 455                 460
ggg atc agc gaa gaa gac gcc atc tcg atg att gtt aac ggt ttc tgc  1440
Gly Ile Ser Glu Glu Asp Ala Ile Ser Met Ile Val Asn Gly Phe Cys
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Claims

1. An inhibitor of *ycf 24* gene product expression and/or activity for use in a method of treatment of the human or animal body by therapy.

2. An inhibitor according to claim 1 for use in a method of treatment of infection by an organism.

3. An inhibitor according to claim 2 for use in a method of treatment of malaria.

4. A method of inhibiting the growth of an organism comprising contacting the organism ex vivo with an inhibitor of *ycf 24* gene product expression and/or activity.

5. A method of identifying a compound that inhibits the growth of an organism comprising
(i) contacting a test compound with the *ycf 24* gene product, and
(ii) determining whether the test compound inhibits the activity of or binds to the product, any such binding or inhibition being indicative that the compound inhibits the growth of the organism.

6. A method of identifying a compound that ~~inhibits the growth of an organism comprising~~
(i) contacting a test compound with a test construct comprising a *ycf 24* promoter operably linked to a coding sequence,
(ii) determining whether the test compound inhibits expression driven by the promoter, any such inhibition being indicative that the compound inhibits the growth of the organism.

7. A method according to claim 5 or 6 in which the organism is a malaria parasite.

8. A compound identified by the method of any one of claims 5 to 7.

9. A compound according to claim 8 for use in the prevention or treatment of an infection by an organism.

10. A method of inhibiting the growth of an organism comprising contacting the organism ex vivo with a compound according to claim 8.

11. Use of a compound according to claim 8 for the manufacture of a medicament for the treatment of an infection by an organism.

12. A pharmaceutical composition comprising a compound as defined in any one of claims 1 to 3 or 8 and a pharmaceutically acceptable carrier or diluent.

13. A method of treating preventing or treating infection by a unicellular organism in a patient comprising administering to the patient an inhibitor as defined in any one of claims 1 to 3 or 8.

14. A method according to claim 13 in which the organism is a malaria parasite.

ABSTRACT

TREATMENT OF INFECTION

An inhibitor of *ycf 24* gene product expression and/or activity is used in a method of treatment of the human or animal body by therapy. In particular the inhibitor is used in a method of treatment of infection by an organism. The organism may be a malaria parasite, such as *Plasmodium falciparum*.

The invention also provides a method of inhibiting the growth of an organism comprising contacting the organism ex vivo with an inhibitor of *ycf 24* gene product expression and/or activity.

The invention further provides a method of identifying a compound that inhibits the growth of an organism comprising
(i) contacting a test compound with the *ycf 24* gene product, and
(ii) determining whether the test compound inhibits the activity of or binds to the product, any such binding or inhibition being indicative that the compound inhibits the growth of the organism.

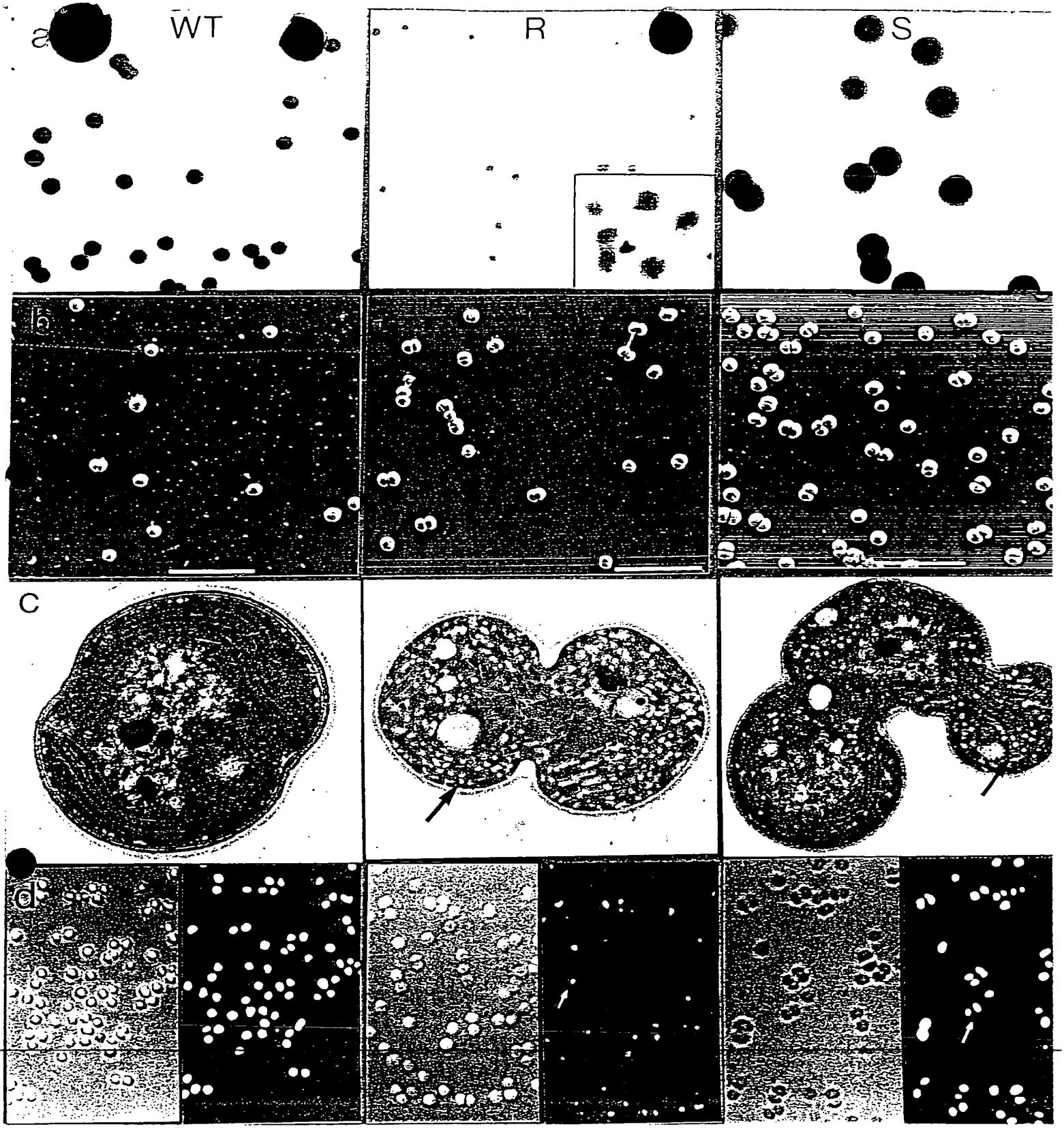

Fig 1 1/1

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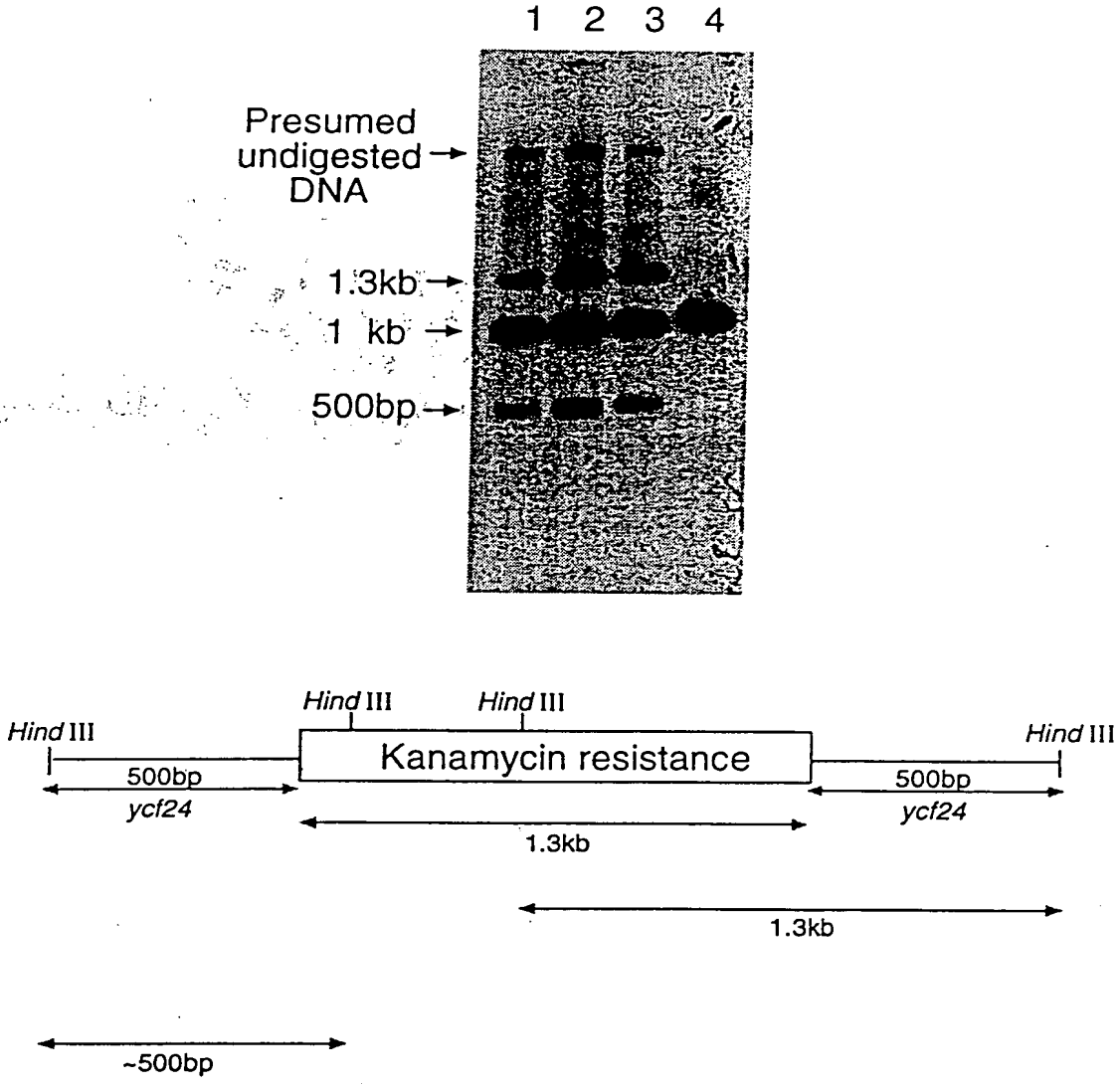
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Hind III
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ycf24
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1.3kb
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Fig 2 2/2